A solvent system for delipidation of plasma or serum without protein precipitation

Bill E. Cham and Brian R. Knowles

Department of Medicine, University of Queensland, Royal Brisbane Hospital, Brisbane, 4029, Australia

Abstract A technique has been developed which attains in 30 minutes complete removal of triglyceride, cholesterol, phospholipid, and unesterified fatty acids from plasma without protein denaturation. Plasma is agitated at room temperature with a mixture of butanol and di-isopropyl ether in a 40:60 (v/v) ratio. The plasma proteins, including the apolipoproteins, remain in solution in the aqueous phase, while the organic phase contains the dissolved lipids. The phases can easily be separated by low speed centrifugation. Different lipids are simultaneously extracted, but the rate of extraction is most rapid for unesterified fatty acids, followed by triglyceride, cholesterol, and phospholipid at, respectively, decreasing rates. Selective extraction of unesterified fatty acids, triglyceride and total cholesterol can be achieved by di-isopropyl ether alone. Ionic strength and pH are not altered by these procedures.

Supplementary key words Di-isopropyl ether · butanol · triglyceride · cholesterol · phospholipid · lipid solvent system · apolipoproteins.

Triglyceride, cholesterol, and phospholipid circulate as complexes of lipid with specific plasma proteins, the apolipoproteins. Past attempts to study these lipid-complexing proteins in whole serum or plasma by extracting the lipid with organic solvents have met with variable success, since such solvent systems tend to denature the protein, or extract the lipid only slowly and incompletely. McFarlane (1) subjected human serum to successive extractions with ether at a temperature range of −20°C to −25°C. However, McFarlane's method failed to achieve complete delipidation, for approximately 35% of the lipid originally in the serum remained with the protein.

Scanu, Lewis, and Bumpus (2) and Scanu (3) applied McFarlane's principles, modified by the use of a mixture of ethanol–ether 3:1 (v/v) to extract lipid from lipoprotein fractions initially separated by ultracentrifugation, and obtained 98% delipidation. Scanu and Schiano (4, 5) also applied the modified technique to whole serum, but obtained protein precipitation, thus introducing the problem of resolubilization. The disadvantages of the Scanu modifications, therefore, are long extraction time, the need to work at low temperatures, the utilization of large quantities of organic solvents, resolubilization of protein, and an end product at an unphysiological pH.

This paper presents a technique for delipidation of serum or plasma, utilizing butanol–di-isopropyl ether (DIPE) in varying proportions to produce a miscible single organic phase. Total delipidation can be achieved within 0.5 hr at room temperature, using small amounts of butanol–DIPE 40:60 (v/v) as the organic phase. When the organic phase is added to plasma a biphasic system results which, after agitation, separates the plasma lipid from the aqueous phase, but leaves the plasma proteins in solution at unchanged pH and ionic strength.

MATERIALS AND METHODS

Outdated human acid citrate–dextrose plasma obtained from the district blood bank was used in the experiments, unless otherwise stated. DIPE was laboratory reagent grade; all other reagents were analytical grade.

Delipidation

Lipid extractions were performed at room temperature in 11 cm × 2.5 cm glass tubes fitted with polyethylene stoppers. Five ml serum or plasma aliquots each containing 0.5 mg of ethylenediamine tetraacetate (EDTA) (to prevent interaction with possible contaminating traces of peroxide); were added to 10 ml of organic phase consisting of DIPE, either with or without butanol. The tubes were then fastened on a blood cell suspension rotator (Clements) to provide end-over-end rotation, at 28–30 rpm, for varying periods of time. After extraction the mixture was centrifuged at 2000 rpm for 2 min to separate the aqueous and organic phases. The aqueous phase was removed, free of organic phase, by careful suction with needle and syringe, and then subjected to quantitative lipid analysis.

Before proceeding to analyses of constituents other than lipid, the delipidated serum or plasma samples were each

Abbreviations: AP, alkaline phosphatase; DIPE, di-isopropyl ether; GOT, glutamate-oxalacetate transaminase; HDL, high density lipoprotein; IgG, IgA, IgM, serum immunoglobulins; LDH, lactate dehydrogenase; LDL, low density lipoprotein; UEFA, unesterified fatty acids.

agitated with two parts of DIPE and then partitioned by low speed centrifugation. The lower aqueous phase was transferred to a vacuum flask by needle and syringe, and residual DIPE removed by extraction with a water pump aspirator at 37°C for 1 min. This wash was undertaken to remove possible traces of butanol, which is slightly soluble in aqueous media. We have found that aqueous solutions containing traces of butanol give positive spectrophotometric readings when proteins are determined by the method of Lowry et al. (6). Butanol contamination also interferes with the estimation of the lactate dehydrogenase (LDH) activity.

Chemical Analysis

Analysis of the lipids in the serum or plasma before and after exposure to the organic solvent was performed by semi-automated Auto Analyzer methods for triglyceride (7), cholesterol (8), phospholipid (9), and unesterified fatty acids (UEFA) (10) which render the lipids totally available for quantitation. The sensitivity of these methods in our laboratory is such that in 1 ml of serum, 10 μg of triglyceride, cholesterol, and phospholipid respectively can be accurately measured. Percentage delipidation was calculated by difference.

Separation of esterified from unesterified cholesterol was attained using a Sephadex LH-20 column according to Cham et al. (11).

Protein was measured by the method of Lowry et al. (6) on a Unicam SP1800 spectrophotometer, using crystalline bovine serum albumin as standard.

Sodium, potassium, total carbon dioxide, total calcium, inorganic phosphate, urea nitrogen, uric acid, creatinine, glutamate–oxalacetate transaminase (GOT), LDH, and alkaline phosphatase (AP) were estimated on a Technicon SMA 12/60 analyzer, using standard Technicon methodology.

α-Amylase activity was determined by the method of Ceska, Birath, and Brown (12), and glucose by the method of Hoffman (13), as modified for use on the Auto Analyzer.

A Radiometer type TTT 1C with a Philips type CA 14/02 electrode was used for pH determinations.

Zonal electrophoresis

Zonal electrophoresis on agarose (14) and cellulose acetate (15) was performed in barbital buffer 0.05 M at pH 8.60. Oil Red O(Gurr) and Coomassie Blue (Sigma) were used to stain lipid and protein, respectively.

Immunoelectrophoresis

Immunoelectrophoresis was performed using 1% precast agarose slides (Corning-Eel, Halstead, Essex, England). The conditions for electrophoresis and immunodiffusion were as described by Keyser (16). Rabbit antisera to human α- and β-lipoprotein were obtained from Behringwerke AG (Farbwerke Hoechst AG, Frankfurt, Main, Germany), each of which gave a single precipitin line with whole serum.

Immunodiffusion

Single radial immunodiffusion applying the method of Mancini, Carbonara, and Heremans (17) was performed for albumin, ceruloplasmin, and transferrin using M-Partigen immunodiffusion plates. IgA, IgG and IgM assays were similarly conducted on Tri-Partigen plates. The plates were supplied by Behringwerke.

RESULTS

When plasma was delipidated with DIPE alone the results shown in **Table 1** were obtained. Incomplete delipidation was observed after 0.5 hr and only UEFA was completely extracted. After 24 hr all triglyceride, 94% cholesterol, but only 4% phospholipid were extracted. UEFA was extracted most rapidly, followed by triglyceride, cholesterol and phospholipid at respectively decreasing rates. When butanol–DIPE 25:75 (v/v) was used total delipidation of plasma, including all the phospholipid, was attained in 3 hr; the rate of removal of triglyceride was similar to that of cholesterol (**Table 2**).

Increasing proportions of butanol in butanol–DIPE extraction mixtures produced increasing degrees of delipidation of human plasma in 1 hr, as illustrated in **Fig. 1**. Triglyceride was extracted more readily than cholesterol, and phospholipid less readily. In 1 hr these lipids were extracted by butanol–DIPE 35:65 (v/v). By changing the respective proportions in the mixture to 40:60 (v/v), total delipidation was attained in 0.5 hr.

Little greater economy in time of attaining complete delipidation was achieved by further increasing the concentration of butanol in the solvent system, or by increasing the volume of the organic solvent with respect to the serum or plasma. High concentrations of butanol in the organic phase

TABLE 1. Delipidation of human plasma by DIPE as a function of time

	Triglyceride		Cholesterol		Phospholipid		Unesterified Fatty Acids	
Extraction Time	Residual	Delipidation	Residual	Delipidation	Residual	Delipidation	Residual	Delipidation
hr	*mg/100 ml*	%	*mg/100 ml*	%	*mg/100 ml*	%	*mEq/l*	%
0	149		154		201		0.47	
0.5	89	40	129	16	201	0	0.00	100
1	45	70	103	33	201	0	0.00	100
4	30	80	82	47	201	0	0.00	100
7	25	83	69	55	197	2	0.00	100
16	12	92	28	82	195	3	0.00	100
24	0	100	9	94	193	4	0.00	100

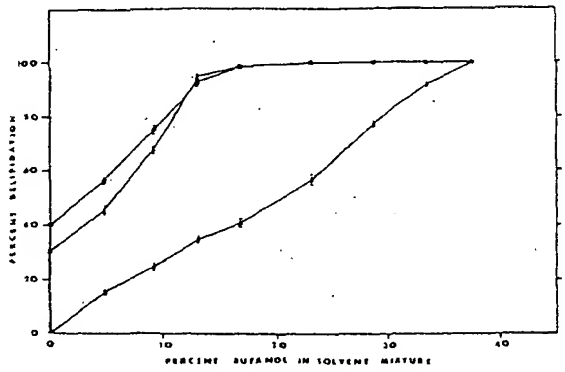

Fig. 1. Delipidation of human plasma in 1 hr by butanol–DIPE mixtures; O, triglyceride; X, cholesterol; Δ, phospholipid. Values are means ± SD of five estimations. Range of initial concentrations (mg per 100 ml of plasma) were: triglyceride, 100–233; cholesterol, 187–290; phospholipid, 180–270.

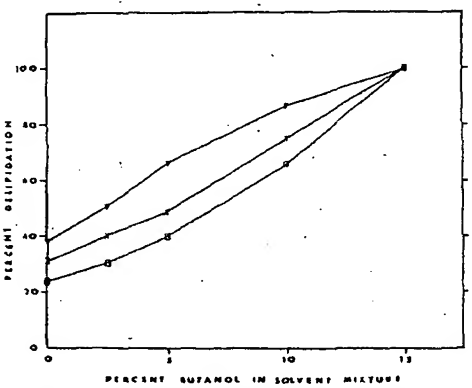

Fig. 2. Extraction of total-, unesterified- and esterified cholesterol in 1 hr by butanol–DIPE mixtures; X, total cholesterol; ∇, unesterified cholesterol; □, esterified cholesterol. Control values (mg per 100 ml of plasma) were: total cholesterol, 205; unesterified cholesterol, 69; and esterified cholesterol, 136.

increase the probability of protein denaturation since visible precipitation of protein occurs when the proportion butanol–DIPE reaches 75:25 (v/v). Small quantities of butanol added to human plasma in the absence of DIPE readily produce protein precipitation. The 1 hr extraction of cholesterol by graduated concentrations of butanol in DIPE was also studied with respect to esterified and unesterified cholesterol, as well as total cholesterol (Fig. 2). Under these circumstances, unesterified cholesterol was extracted more readily than esterified cholesterol.

Two aliquots of plasma which were shown quantitatively to contain no lipid after 0.5 hr exposure to butanol–DIPE mixture 40:60 (v/v), were subjected to electrophoresis on agarose gel, together with two aliquots of the control (unextracted) plasma (Fig. 3). One control and one delipidated sample (B & A, respectively) were stained for protein with Coomassie Blue, and the remaining control and delipidated strips (C & D, respectively) were stained for lipid with Oil Red O. No lipid stainable material was demonstrated in the delipidated sample, D. Comparison of the protein-stained strips A & B revealed a slight broadening of the α-globulin band in the delipidated sample, A.

On immunoelectrophoresis untreated serum samples produced single precipitin reactions with anti-LDL and with anti-HDL (Fig. 4); the delipidated serum produced a single precipitin reaction with anti-LDL which migrated faster than its parent untreated LDL under the same electrophoretic conditions. The precipitin reaction of delipidated serum and anti-HDL was more complex. Here, the delipidated HDL migrated more slowly than its parent untreated HDL; in addition there was a double precipitin reaction of the delipidated serum with anti-HDL.

In order to demonstrate that no protein was extracted by the organic solvent, five serum samples were exposed to the butanol–DIPE 40:60 (v/v) mixture for 0.5 hr, and 6 ml of the organic phase were then pipetted from each sample, care being taken to avoid the aqueous solvent interphase. The solvent from the five aliquots of organic phase was evaporated, and each residue was dissolved in 0.2 ml of diethyl ether followed by the addition of 1 ml of 0.1 M sodium hydroxide, and the mixture was agitated. After 2 min centrifugation at 1500 rpm the diethyl ether phase was removed by aspiration. When the remaining alkaline phases were subjected to protein analysis (6) each yielded a spectrophotometric reading equivalent to the blank control, thus indicating that no protein had been extracted by the original organic solvent.

Table 3 details the concentrations of total protein, albumin, ceruloplasmin, transferrin and three immunoglobulins in a

TABLE 2. Delipidation of human plasma by butanol–DIPE 25:75 (v/v) as a function of time

Extraction Time	Triglyceride		Cholesterol		Phospholipid		Unesterified Fatty Acids	
	Residual	Delipidation	Residual	Delipidation	Residual	Delipidation	Residual	Delipidation
hr	*mg/100 ml*	*%*	*mg/100 ml*	*%*	*mg/100 ml*	*%*	*mEq/l*	*%*
0	164		205		217		0.47	
0.25	25	85	25	88	130	40	0.00	100
0.50	15	91	14	93	102	53	0.00	100
0.75	8	95	8	96	91	58	0.00	100
1	7	96	6	97	78	64	0.00	100
1.5	5	97	4	98	48	78	0.00	100
2					11	95	0.00	100
3	0	100	0	100	0	100	0.00	100

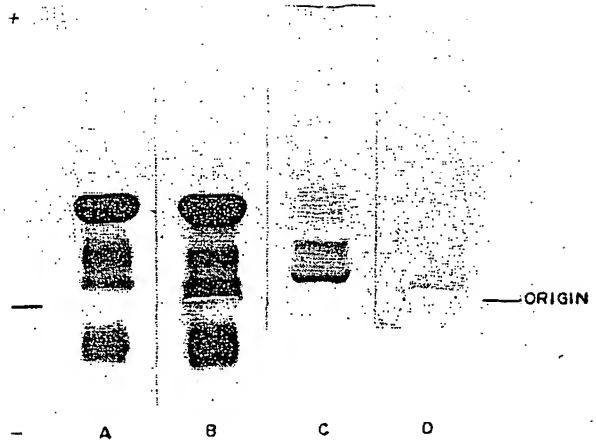

Fig. 3. Electrophoretic strips comparing control (unextracted) human plasma with human plasma extracted with butanol–DIPE 40:60 (v/v) for 0.5 hr. A and D, delipidated plasma; B and C control plasma; A and B, Coomassie Blue stain for protein; C and D, Oil Red O stain for lipid.

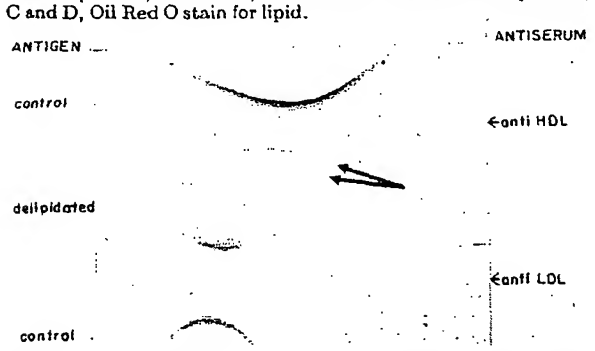

Fig. 4. Immunoelectrophoresis of control and delipidated whole serum lipoproteins on agarose gel. Antibody troughs contained antisera to human HDL or LDL. Antigen wells contained whole control serum or delipidated whole serum. The arrows indicate the two arcs present only when lipoproteins from delipidated serum reacted against anti-HDL. Migration was from left to right. Proteins were stained with Coomassie Blue.

representative sample of fresh nonfasting human serum without added EDTA before and after exposure to butanol–DIPE 40:60 (v/v) for 0.5 hr. The activities of AP, α-amylase, GOT and LDH are also tabulated, as are pH and other ionic and chemical constituents.

DISCUSSION

A variety of organic solvents are widely used for the extraction of lipid from plasma or serum in quantitative estimations, but most of these cause protein denaturation, often irreversible. Those solvent systems which do not irreversibly denature protein, and are therefore useful for qualitative studies, extract lipid only slowly and incompletely (1), and either introduce problems of protein resolubilization or resultant solutions at unphysiological pH (2–5). DIPE has been shown to remove triglyceride and cholesterol slowly and almost completely, but scarcely extracts any phospholipid. This observation provides a methodology leaving intact lipoprotein phospholipid in the presence of little cholesterol and no triglyceride. Butanol, a powerful lipid solvent, causes precipitation of plasma and serum proteins when used alone as a lipid extraction medium.

The present studies demonstrate that mixtures of butanol and DIPE can be utilized to extract triglyceride, cholesterol, phospholipid, and UEFA, rapidly, and simply from plasma or serum samples, with otherwise minimal change in the physical state and the chemical constituents of the extracted sample (Table 3). Since the Auto Analyzer methods are sufficiently sensitive to measure 10 μg of triglyceride, cholesterol, and phospholipid in 1 ml of plasma, the maximal amount of each of these lipids remaining in the 5 ml delipidated sample cannot exceed 50 μg, and may well be less. This represents at least 99.6% delipidation of those samples containing the greatest concentrations of lipids. The rate of extraction is dependent upon the proportion of butanol present (Fig. 1). Within the range of triglyceride, cholesterol and phospholipid concentrations studied, the percentage of each of these lipids extracted in a given time and with a fixed proportion of

TABLE 3. Quantitative comparison of serum constituents before and after delipidation of fresh human serum from non-fasting subjects

		Control	Delipidated			Control	Delipidated
Sodium ion	mM/L	144	143	GOT	IU	25	23
Potassium ion	mM/L	5.2	5.1	AP	IU	81	80
Chloride ion	mM/L	104	105	LDH	IU	213	213
Total CO_2	mM/L	28	28	α-amylase	IU	283	283
Creatinine	mg/100 ml	1.3	1.3	Glucose	mg/100 ml	92	91
Uric acid	mg/100 ml	6.8	6.7	IgA	mg/100 ml	168	167
Urea nitrogen	mg/100 ml	18	17	IgM	mg/100 ml	144	144
Inorganic phosphate	mg/100 ml	4.1	4.1	IgG	mg/100 ml	1402	1395
Calcium	mg/100 ml	9.5	9.6	Ceruloplasmin	mg/100 ml	30	31
Total cholesterol	mg/100 ml	192	0	Transferrin	mg/100 ml	354	354
Triglyceride	mg/100 ml	204	0	Albumin	g/100 ml	5.12	5.12
Phospholipid	mg/100 ml	218	0	Total Protein	g/100 ml	7.35	7.42
Unesterified fatty acid	mEq/L	0.78	0.00	pH		7.37	7.37

butanol in the extraction medium is independent of the initial absolute concentration of each lipid (Fig. 1). Extraction of the three lipids is not uniform, phospholipid being extracted less readily than either triglyceride or total cholesterol. Similarly, esterified cholesterol is extracted less rapidly than free cholesterol (Fig. 2).

Scanu and Edelstein (18) have drawn attention to the extraction of small quantities of protein from aqueous solutions of purified lipoprotein fractions by various organic solvents. Neither whole serum or plasma, nor the solvents used in the present studies were investigated by these authors but, in recognition of their observations, samples of the organic phase were analyzed for protein concentration following the delipidation process. No detectable protein was found in the butanol–DIPE 40:60 (v/v) mixture after 0.5 hr.

The increased width of the α-globulin band of the totally delipidated sample subjected to zonal electrophoresis, when compared with that of the undelipidated sample (Figure 3, A and B), indicates a change in electrophoretic mobility following delipidation. Changes in electrophoretic mobility as a result of delipidation are also apparent on immunoelectrophoresis, the precipitin arc produced by the delipidated serum when exposed to LDL-antibody being in advance of that produced by undelipidated serum under the same conditions. Reaction of the same serum samples with HDL-antibody shows that the precipitin arc produced by the delipidated sample occurs slightly behind that produced by the control serum. This latter observation is in accordance with that of Sodhi and Gould (19) who have shown that apoHDL is electrophoretically less mobile than HDL. Levy and Frederickson (20) studied partially delipidated HDL and similarly observed a slower migration rate than the undelipidated control on immunoelectrophoresis. In addition their delipidated sample yielded two immunoreactive components, with differing migration rates. In the current experiments, immunoelectrophoresis of the delipidated serum against anti-HDL yielded a dual crescent (Fig. 4), suggesting molecular heterogeneity of the delipidated HDL. These observations indicate qualitative changes in lipoprotein following delipidation, but do not conclusively indicate whether there may have been any quantitative change affecting immunoreactivity.

Since no protein was recoverable from the organic phase after the delipidation procedure, and since quantitative immunodiffusion analysis of the residual aqueous phase for albumin, immunoglobulins, ceruloplasmin and transferrin (Table 3) revealed no loss of these proteins when compared with the control serum, it may be concluded that no significant denaturation of protein occurs. This conclusion is reinforced by observation of the activities of GOT, AP, LDH, and α-amylase (Table 3) in the control and delipidated samples, where no appreciable differences in activity were detected. Since enzymically active proteins lose their catalytic activity on denaturation and since the activity of such molecules is determined by the juxtaposition or interaction of amino acid side chains present in the native conformation, it can be deduced that for the enzymes studied there was no denaturation after delipidation.

The similarity of pH, concentration of ionic constituents, and concentrations of creatinine, glucose, and urea (Table 3) in the undelipidated and delipidated samples indicate that the delipidated proteins remain in a more physiological environment than has been achieved using the previously available delipidation techniques.

Although this short and simple delipidation technique has been applied to whole plasma and serum, optimal conditions being 0.5 hr exposure to butanol–DIPE 40:60 (v/v), it raises the prospect of separating apolipoprotein moieties from lipoprotein without apparent damage, thus affording opportunities of establishing the functional capacities of the plasma apolipoproteins.

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